Salt Tolerance Conferred by Overexpression of a Vacuolar Na\(^+\)/H\(^+\) Antiport in Arabidopsis

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Agricultural productivity is severely affected by soil salinity. One possible mechanism by which plants could survive salt stress is to compartmentalize sodium ions away from the cytosol. Overexpression of a vacuolar Na\(^+\)/H\(^+\) antiport from Arabidopsis thaliana in Arabidopsis plants promotes sustained growth and development in soil watered with up to 200 millimolar sodium chloride. This salinity tolerance was correlated with higher-than-normal levels of AtNHX1 transcripts, protein, and vacuolar Na\(^+\)/H\(^+\) (sodium/proton) antiport activity. These results demonstrate the feasibility of engineering salt tolerance in plants.

Salinity stress is one of the most serious factors limiting the productivity of agricultural crops. The detrimental effects of salt on plants are a consequence of both a water deficit resulting in osmotic stress and the effects of excess sodium ions on critical biochemical processes (1). In order to tolerate high levels of salt, plants should be able to utilize ions for osmotic adjustment and internally distribute these ions to keep sodium away from the sites of metabolism (1). Plant cells are structurally well suited for the sequestration of ions because of the presence of large, membrane-bound vacuoles. It has been proposed that in salt-tolerant plants, the compartmentation of Na\(^+\) into vacuoles, through the operation of a vacuolar Na\(^+\)/H\(^+\) antiport, provides an efficient mechanism to avert the deleterious effects of Na\(^+\) in the cytosol and maintains osmotic balance by using Na\(^+\) (and chloride) accumulated in the vacuole to drive water into the cells (2). This Na\(^+\)/H\(^+\) antiport transports Na\(^+\) into the vacuole by using the electrochemical gradient of protons generated by the vacuolar H\(^+\)-translocating enzymes, H\(^+\)-adenosine triphosphatase (ATPase) and H\(^+\)-inorganic pyrophosphatase (PP\(_{ase}\)) (3, 4). Vacular Na\(^+\)/H\(^+\) antiport activity was shown first in tonoplast vesicles from red beet storage tissue (5) and later in various halophytic and salt-tolerant glycophytic species (6, 7). Chloride transport into the vacuole is mediated by anion channels (8). In Arabidopsis, a vacuolar chloride channel, At-CLC\(_{D}\), similar to the yeast GefI, has been cloned (9). The analysis of genes involved in cation detoxification in yeast led to the identification of a novel Na\(^+\)/H\(^+\) antiport (Nhx1). Nhx1 was localized to a prevacuolar compartment and showed a high degree of amino acid sequence similarity to Na\(^+\)/H\(^+\) antiports from Caenorhabditis elegans and humans (NIH6, mitochondrial) (10). Recently, the Arabidopsis thaliana genome-sequencing project has allowed for the identification of a plant gene (AtNHX1) homologous to the Saccharomyces cerevisiae Nhx1 gene product (11–13). Both Nhx1 and GefI are localized to the yeast pre-vacuolar compartment, suggesting a role for this compartment in salt tolerance. Overexpression of AtNHX1 suppresses some of the salt-sensitive phenotypes of the ahl1 strain (13), suggesting that the plant and the yeast gene products might be functionally similar. AtNHX1 transcripts are found in root, shoot, leaf, and flower tissues (11). To determine the subcellular localization of AtNHX1, we immunoblotted membrane fractions (14) isolated from wild-type plants and plants overexpressing AtNHX1 (15) with antibodies raised against the COOH-terminus of AtNHX1 (Fig. 1). A protein of an apparent molecular mass of 47 kD was detected mainly in the tonoplast- and Golgi/endoplasmic reticulum (ER)—enriched fractions, and was more abundant in the transgenic plants. No noticeable cross-reactivity

References and Notes
8. We observed a qualitatively similar phenomenon with the nondeuterated oligomers OS-OEP. In that system, the OEP-rich phase aligns at the air interface, and the OS-rich phase aligns at the gold surface.
9. We are grateful to L. J. Fetter's and M. Wilhelm for kindly donating the OEP and dOEP oligomers. We thank J. F. Joanny, S. Safran, and H. Groll for enlightening discussions and the U.S.-Israel Binational Science Foundation (grant 95-147), the Wolfson Family Charitable Trust (to R.Y.-R.), the German-Israel Foundation (grant I-568-275/0297), and the Israel Ministry of Science (Taishiot Program) for support.
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\[ \text{Na}^{+}/\text{H}^{+} \]
with these antibodies was observed in plasma membrane– and mitochondria-enriched fractions, which suggests that the plant NHX1 does not share the same subcellular localization as that determined for NHE6 (16). It remains unclear whether the immunoreactive protein observed in the Golgi/ER-enriched fractions represents contamination of this fraction with tonoplast proteins (7) or presence of the target protein in prevacuolar compartments. Nonetheless, immunoblots of purified leaf vacuoles from wild-type plants (Fig. 1B). The apparent molecular mass (47 kD) of AtNHX1 is somewhat lower than that predicted by the amino acid sequence of the AtNHX1 open reading frame (58 kD) and may reflect anomalous migration in the SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel (18) or specific cleavage (or degradation).

In order to assess whether AtNHX1 provides a Na+/H+ exchange function, Na+-dependent H+ movements (19) were measured in vacuoles isolated from the leaves of wild-type plants and plants overexpressing AtNHX1 (Fig. 2). The Na+/H+ exchange rates were very low in vacuoles from wild-type plants (Fig. 2A, trace 1). In contrast, Na+/H+ exchange rates were much higher in vacuoles from the transgenic plants (Fig. 2A, trace 2). The Na+/H+ antiport activity was not affected by the presence of 30 mM K+ ions in the assay medium (20), indicating selectivity for Na+.

Electroneutral Na+/H+ exchange is suggested by the similar rates of Na+-dependent H+ movements obtained when the membrane potential was clamped by the addition of 1 μM valinomycin and 2 mM K+ to the assay medium (20). The Na+ dependence of the H+ flux is evidence of a Na+/H+ antiport mechanism. The Na+/H+ exchange displayed Michaelis-Menten kinetics with respect to extravacuolar Na+ concentrations (Fig. 2B). Similar apparent Km values of the exchanger for Na+ were obtained with two independent transgenic lines, Km = 7 mM (Fig. 2C) and 6.1 mM (20). These values are of the same order of magnitude as those reported for other plant species (7). The relative increase in protein abundance (Fig. 1B) is less than the increase in Na+/H+ antiport activity measured in vacuoles from transgenic plants (Fig. 2, A and B). These observations suggest that in wild-type plants under normal growth conditions, AtNHX1 function may be repressed. Overexpression of AtNHX1 may overcome this endogenous repression mechanism.

Salt tolerance was tested in wild-type and transgenic plants overexpressing AtNHX1 (Fig. 3). Wild-type plants displayed progressive chlorosis, reduced leaf size, and a general growth inhibition when watered with a NaCl-containing solution. These inhibitory effects increased progressively with the increasing NaCl concentration in the watering solution. The transgenic plants were unaffected by up to 200 mM NaCl (Fig. 3, lower panel) and plant development was not compromised, as the transgenic plants bolted and set seed in all salt treatments. However, transgenic plants grown at 300 mM NaCl displayed a reduction in leaf size and chlorosis (20). The three independent transgenic lines showed a similar increase of AtNHX1 transcript levels (Fig. 3, inset) and similar salt tolerance (20). The Na+ content of both wild-type and transgenic plants increased with exposure to high NaCl (Fig. 4). The higher Na+ content of the transgenic plants growing in 200 mM NaCl, together with the sustained growth (Fig. 3) and reduced leaf size (Fig. 3), suggests that AtNHX1 is involved in Na+ exclusion.
Fig. 3. Salt treatment of wild-type plants and plants overexpressing AtNHX1. Transgenic T3 plants from the lines overexpressing AtNHX1 were used in assessing salt tolerance. Twenty wild-type plants and 20 of each of the three lines of transgenic plants overexpressing AtNHX1 were grown on a short-day cycle (8 hours light, 16 hours dark). Each set of 20 plants was divided into five groups (labeled A through E) of four plants each. We applied 25 ml of a diluted nutrient solution (1/8 MS salts) every other day over the 16-day watering treatment. The control group received no NaCl supplementation. The remaining groups were watered without nutrient solution supplementation with NaCl. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 4 days for each group, to the indicated maximum: (A) control; (B) 50 mM NaCl, (C) 100 mM NaCl, (D) 150 mM NaCl, and (E) 200 mM NaCl. The salt-tolerance phenotype was observed in three independent transgenic lines tested. The transgenic line shown (2) is representative of the three tested lines as are the plants from each treatment group. (A) (upper panel) Wild-type plants. (B) (lower panel) Plants overexpressing AtNHX1. [Inset] Northern blot of RNA isolated from leaves of wild-type (wt) and three independent lines (2’, 3’, 4’) of transgenic plants grown in the absence of NaCl. RNA was probed with AtNHX1 cDNA. Equal amounts of total RNA were present in each sample (30 μg). In all plants, the endogenous 2.1 kilobase (kb) transcript was detected. An additional transcript of ~1.7 kb that corresponds to the predicted open reading frame of AtNHX1 can be seen only in the transgenic plants. Apparent molecular size (kb) of the transcripts is indicated to the left.

Fig. 4. The Na⁺ content in wild-type (black bars) and transgenic plants (white bars) grown in the absence or presence of 200 mM NaCl. The aboveground parts of the plants were harvested at the end of the salt treatment. Dry weight was measured after 24 hours at 70°C, and Na⁺ content was determined by atomic absorption spectrophotometry. Values are the mean ± SD (n = 4).

The increased vacuolar antiport activity (Fig. 2), is consistent with increased vacuolar compartmentation of Na⁺ in the transgenic plants. We did not detect an increase in AtNHX1 transcript levels in response to NaCl (50 to 250 mM) (13) or upon the application of exogenous ABA (20). We have analyzed RNA from young seedlings and mature plants, and from the roots, shoots, and leaves of NaCl-stressed plants at different time points (6 to 36 hours) grown in petri dishes or in soil. No increase in the AtNHX1 protein product was detected either by immunoblotting or by vacuolar Na⁺/H⁺ activity assays in response to NaCl-stress (20). These results would suggest that the induction of AtNHX1 protein synthesis or vacuolar Na⁺/H⁺ antiport activity in response to NaCl-stress in Arabidopsis wild-type plants requires conditions which are currently unknown. Since Arabidopsis is a glycoplastic plant with a sensitivity to salt similar to most crop plants, our findings suggest the feasibility of genetic engineering crop plants with improved salt tolerance.

References and Notes
14. Membrane proteins were purified from 5-week-old Arabidopsis thaliana plants using the method described (5) with the following modifications. Tono-plastid, Golgi/ER, and plasma membrane-enriched fractions were obtained from the 0%/20%, 20%/30%, and 30%/40% interfaces, respectively. Chymostatin, leupeptin, aprotinin, and pepstatin (each to a 10 μg final concentration) were added to the resuspenion buffer.
15. The open reading frame sequence obtained by our lab is identical to that recently described by Gaviola et al. [13]. The AtNHX1 putative open reading frame was amplified by polymerase chain reaction (PCR) from the cDNA template and cloned into the SalI/Sma I sites of PBSN1 (S. B. Narasimhulu, X. Deng, R. Sarria, S. B. Gelin, Plant Cell 8, 873 (1996)) in a sense orientation using the following primers: 5′-ATX1-5′-CCGGATCCCCGACCATGTCTGAGCTCTGC-3′ and 5′-AUXCT2-5′-CCAGTTTCGGAACGTCCTTGTCC-3′. The resulting vector contained AtNHX1 under the control of the supermosa promoter. Agrobacterium-mediated transformation was performed using flowering plants with primary roots reaching 15 cm. Plants were dunked into a bacterial solution, for 5 min, containing 0.5% Murashige and Skoog (MS) salts; 0.5% gellan; 5% sucrose and 0.03% Silwet. The same procedure was repeated 12 days later. Transgenic plants were selected by plating the seeds on 0.5% MS agar plates containing 25 mg/liter kanamycin. Plants were carried for two more generations in order to identify plants homozygous for the transgene.
17. Vacuoles were isolated by osmotic lysis of leaf protoplasts and floated on top of a 2% Ficol cushion as described [19].
20. M. P. Aps, G. S. Aharon, E. Blumwald, data not shown.
21. Antibodies were raised in rabbits against a glutathione S-transferase (GST)–fusion protein of the COOH-terminal region of AtNHX1. These fusion proteins were produced in Escherichia coli (strain BL21pLysS) that had been transformed with the pGEX2TK, into which we had subcloned the region encoding the final 95 amino acids of AtNHX1. The Eco RI– Bam HI fragment of the PCR product amplified from the cDNA template using the PCR primers, forward–5′-CCGGATCCCCCGACCATGTCTGAGCTCTGC-3′ and reverse–AUXCT2 (15) was ligated into the same sites on the pGEX2TK vector. Induction and purification of the recombinant GST-fusion was as be performed manufacturers instructions (Pharmacia). Antibodies were purified by first passing the antiserum over an immobilized GST column (Pierce), and then by affinity blot purification [E. Harlow and D. Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press, New York, 1988), p. 460].
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